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A MICRO LAGOON TECHNIQUE FOR THE CULTURE AND OBSERVATION OF ISOLATED MAMMALIAN CELLS

by Clarence D. Cone, Jr., and Kathryn H. Peddrew

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A MICRO LAGOON TECHNIQUE FOR THE CULTURE AND OBSERVATION OF ISOLATED MAMMALIAN CELLS[1]

By Clarence D. Cone, Jr., and Kathryn H. Peddrew
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SUMMARY

A technique for physically partitioning a monolayer of cells into micro regions on a culturing surface is described. The method consists of forming a thin grease layer into a field of tiny ponds or lagoons in which small numbers of cells or other microbiological specimens can be confined for purposes of growth, manipulation, and microscopic observation. Special instruments and procedures which have been developed for lagoon-field formation are described in detail, as are the characteristics of various formation materials which have been evaluated. Factors and problems affecting various aspects of lagoon-field suitability for cytological purposes are discussed, and the details of special culturing vessels and lagoon-inoculation procedures which have been developed by the authors for particular types of experiments are presented. A number of cytological applications, particularly in the field of time-lapse cinephotography, in which the technique has proven to be exceptionally valuable are outlined. The basic micro lagooning techniques described have considerable flexibility for adaptation and should thus prove to be useful for a wide range of cytological applications where simple micro isolation is a prime requirement.

INTRODUCTION

In many cytological investigations it is either desirable or necessary to deal with individual cells or with small groups of cells in monolayer culture. A typical example, for instance, is the development of a micro clone from a single parent cell. (See ref. 2.) Since it is very difficult to maintain small numbers of cells in culture, it is usually necessary in such cases to begin with a well-populated monolayer and to select test specimens from those areas of the field having a sufficiently sparse density of cells. This procedure

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[1]A summary of some of the principal techniques discussed in this paper was presented in an address delivered to the Medical Sciences Section of the Virginia Academy of Science's Annual Symposium, May 5, 1967, Norfolk, Virginia; an outline of the basic concept and technique has been published in reference 1.

has serious drawbacks, however, in that the continuous migration and mitoses of cells within the test area make long-term observation and identification of particular experimental specimens difficult, if not impossible. For such investigations it is obviously desirable to have some means for physically partitioning a large field of cells into micro regions without restricting the free access of the common culture medium to all parts of the field.

The purpose of this paper is to describe an effective technique for obtaining such micro partitioning in a simple and reproducible manner by forming a field of tiny ponds or lagoons directly on the surface of a suitable culturing vessel. The procedures for forming such lagoon fields are discussed herein in some detail, as are the principal factors affecting the size, shape, and stability of the individual lagoons. Techniques which have been developed for inoculating the lagoons with cells for particular experimental studies are also described, along with some features of the observed behavior of cells confined to micro lagoons. Finally, a brief outline is presented of some specific experimental applications in which the method has been highly useful.

The basic micro lagoon techniques and instruments described herein were originally developed by the senior author at the Langley molecular biophysics laboratory (MBL). Their primary use has been in studies of electrophysical mechanisms of cell mitosis.

LAGOON FORMATION TECHNIQUES

Basic Procedure

The basic technique, as illustrated in figure 1, consists in applying under sterile conditions a thin coating of a suitable nontoxic grease to the smooth end of a small solid cylinder, by using a fine brush or by simply coating the cylinder end directly from a small glass syringe. The coated end is pressed firmly against the surface of the desired culturing vessel (for example, a Petri dish or perfusion chamber cover glass) and oscillated slightly to obtain a uniform contact of the grease with the surface. The cylinder, which is termed the "formation signet," is then lifted rapidly from the surface by applying a strong force in the direction of the cylinder axis. Due to the cohesion of the grease, this separation results in the polymeric material being pulled into a fine meshwork of connected ridges. Very small holes are left in the thin grease layers of many of the flat areas enclosed by the ridge network, and the grease sheet surrounding these holes contracts immediately after formation; thus, the openings are enlarged and the free surface area is exposed. This overall action produces a field containing large numbers of small lagoons in which the walls consist of the grease material and the bottoms consist of free culturing surface.

Within any one field (equal in extent to the area of the formation signet face), a wide range of lagoon shapes and sizes is usually found. Some typical lagoon formations obtained in this manner are illustrated in figure 2. Because it results in the formation of a continuous field of small lagoons on the monolayering surface, this process has been designated the "micro lagoon technique." The lagoons so formed are subsequently inoculated with cells (fig. 2, micrograph B) for test purposes.

Formation Signets and Special Techniques

The size of the depressions within the initial ridge network, and hence the size distribution of the resulting lagoons, is governed in considerable measure by the rapidity with which the signet is separated from the vessel surface during formation and by the parallel alinement of the signet face and the vessel surface at separation. The greased signet used in preparing the lagoon field adheres very firmly to either plastic or glass upon pressing, and a firm grip is required to lift it normally from the surface with the desired rapidity. A series of signets (fig. 3) which have been specially designed for specific types of lagoon field formation are described subsequently along with some of the techniques employed in their use.

Flat-faced signet.- The simple flat-faced signet shown in figure 3(a) is made of stainless steel and is provided with a grooved finger-handle for accurate manipulation. This signet is the most commonly used type. The size of the total lagoon field can be varied by using signets with different face diameters. A family of such flat-faced signets having various diameters is shown in figure 3(c).

A technique which has been found useful for obtaining reproducible lagoon fields with flat-faced signets consists in dipping the body of a stainless-steel signet in absolute ethanol and then igniting the alcohol as in normal sterilization. The resulting heating of the signet is sufficient to assure that when it is subsequently touched to a surface of the formation grease, a uniform and reproducibly thin layer of the softened material adheres to the signet face.

Ring-faced signet.- The ring-faced signet shown in figure 3(b) is used for creating a circular boundary of grease between two different cell fields. The ring is used to form a high circular ridge of grease on the culturing surface, and the interior and exterior areas are then inoculated with cells of the desired type(s). These cells may be of either the same or of different lines. In effect, the ring provides an isolated cultured field of macro dimensions. The manner in which such a ring field is inoculated with cells is discussed in the section "Inoculation Procedures."

Grid-faced signet.- The individual lagoons formed within the field of a flat-faced signet are randomly distributed and, if very small, may be somewhat difficult to locate easily under the microscope, especially if tests are being conducted with cells in several

such lagoons within the same field. The grid-faced signet pictured in figure 3(d) was developed for producing a relatively uniformly spaced grid of micro lagoons in cases where such a field is desirable. The signet face is drilled with a rectangular array of circular holes, each with a diameter of 0.004 inch; these holes serve as initiating centers for lagoon formation and thus produce a field with more or less evenly spaced lagoons. Figure 4 is a photomicrograph of a typical field produced by this signet.

To insure uniform conditions during the formation of the gridded fields, the grid-faced signet is made the center plunger of a spring-loaded barrel arrangement, as sketched in figure 5. The signet face is initially coated by pressing it against a uniform grease film spread on a flat surface (for example, on a microscope slide). The plunger-and-barrel unit is then placed over the culturing surface and the plunger depressed until it makes firm contact with the surface. Upon release, the compressed spring quickly raises the plunger perpendicularly from the surface. By use of a relatively stiff spring, a high degree of spatial regularity of lagoons in the gridded fields is obtained.

FACTORS AFFECTING LAGOON SIZE, SHAPE, AND STABILITY

General Factors

The principal characteristics of lagoons which are important for most experimental work are the size and depth, and, in some cases, the shape. The durability of the lagoon field is also important for relatively long-term tests. Formation-material properties, the nature of the culture vessel surface, and the formation technique are important in obtaining the desired lagoon characteristics. In the case of greases, viscosity and adhesion (or surface tension) properties are especially important. The viscosity or stiffness determines in a large measure the size, distribution, and depth of the lagoons formed, the less viscous greases appearing to provide initially smaller lagoons. The adhesion determines the stability of initial lagoon size and shape, as well as durability of the field as a whole. Adhesion depends upon both the nature of the grease and the culturing surface.

Most greases adhere well to both glass and plastic surfaces in the dry state. When the lagoon field is immersed in a warm culture medium, however, some greases because of their hydrophobic nature become highly unstable and tend to release from the vessel surface, especially on glass. This condition results ultimately in a degeneration of the initial lagoon field into a group of isolated grease blobs or droplets, or a separation of the entire field from the surface. Other greases, however, maintain very stable formations under culture media, even on glass.

Other materials besides greases have been investigated for lagoon formation suitability. For the most part, these materials have been permanently hardening materials,

such as cements or dopes. A summary of the characteristics of these materials and those of the greases which have been investigated is presented in the following sections.

In addition to the physical properties outlined previously, a suitable lagoon formation material must also be nontoxic to the cells under study and either initially sterile or capable of sterilization. Most greases can be sterilized in closed containers by normal autoclaving procedures. Since most greases melt at autoclave temperatures, however, it is not possible to sterilize the preformed lagoon field; the fields must be prepared aseptically. Because of the chemical nature of some of the permanently hardening materials, they may be biologically uncontaminated as commercially packaged. The toxicity of a given material for a particular cell line must generally be checked experimentally.

Lagoon Formation Materials

Greases.- Several greases or grease-like materials have been evaluated for lagoon formation suitability. These materials include silicone vacuum grease, white petroleum jelly, hair control wax, lubricating grease, and common lard. All of these materials formed fields having lagoons of adequate size for cell culture. However, the most generally suitable materials were found to be the silicone grease and the hair control wax and these materials are now used almost exclusively in all culture work at the MBL. Consequently, subsequent discussion is limited to these two materials.

Dow Corning silicone grease (DCS).- This material (Dow Corning high vacuum grease silicone lubricant) is a product of the Dow Corning Corporation, Midland, Michigan, and is hereinafter referred to as DCS. DCS forms excellent lagoon fields for many purposes and is nontoxic, at least for the three cell lines tested (that is, mouse fibroblasts (L-cell line, National Cancer Institute), human adult liver (No. 71-106), and human embryonic skin (72-176)).[2] Unfortunately, DCS lagoon fields are only moderately stable on plastic surfaces and are very unstable on glass, the lagoons usually tending to enlarge continuously after formation and immersion in culture medium. Occasionally, however, DCS fields on glass will ultimately (that is, after about 24 hours) reach a stable condition, and thus yield very large lagoons which are desirable for certain types of experiments. The height of the lagoon walls in such cases also increases as the lagoons enlarge; this condition is an advantage where deep lagoons are necessary. With proper formation techniques, adequately small DCS lagoons can sometimes be obtained; these lagoons are usually surrounded by a large grease area which apparently provides the necessary stability.

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[2]Numbers refer to the catalog classification of the Microbiological Associates, Inc., Bethesda, Maryland.

A condition where the adhesion instability of DCS is particularly undesirable occurs when the field is used with thin perfusion chambers. Here the high surface tension of the medium meniscus during filling of the chamber by syringe often peels the entire field from the surface. This condition can usually be avoided, however, by filling the chamber (at least partially) before assembly.

Figure 6 presents views of two typical DCS lagoon fields formed on plastic. The average lagoon diameter in such DCS fields when formed on plastic with the large (1/2-inch-diameter) flat-faced signet of figure 3(a) is approximately 120 microns (μ) with a range of 20 to 500 microns (μ). The upper limit depends on the relative stability of the particular formation and may, on glass, reach almost the full 1/2-inch (1.27 cm) diameter of the field.

Hair control wax (HCW).- In a search for materials having better adhesion to glass under culturing conditions, it was found that wax-containing greases produced especially stable lagoons. The unperfumed base of a hair dressing preparation (Barbers Butch Wax, a product of the Stephan Company, Ft. Lauderdale, Florida[3]) was found to have excellent lagoon formation and stability properties. This material, designated herein as HCW, adheres very firmly to glass as well as plastic and shows little tendency to release or to alter lagoon size or shape under warm culture media. In some cases, however, the grease rollback in the initial lagoon formation leaves tiny spheres of HCW on the surface so that lagoons of HCW are often not as "clean" as those of DCS. This grease debris however is not always present in HCW lagoons. Figure 7 pictures two typical lagoon fields of HCW formed with the same flat-faced signet as used for the DCS fields of figure 6.

Permanently hardening materials.- Several permanently hardening materials were investigated for obtaining more rigid and permanent lagoon fields. Among these materials were such substances as model airplane cement, white shellac, and clear lacquer. These materials were generally unsatisfactory for the following reasons. In some cases the solvent of the formation material attacked plastic culturing surfaces so that even when lagoons were subsequently formed, the exposed culture surface had been rendered semi-opaque or otherwise unsuitable optically. In others, the formation material when applied to either plastic or glass so wetted the surface that it failed to release at all even though good lagoon walls (ridges) were produced by the signet. The resulting optical aberrations made such lagoons unusable. In the case of clear lacquer, excellent rigid lagoons with deep walls and clear bottoms were obtained on glass; however, the hardened lacquer plaque swelled slightly upon subsequent immersion in culture medium and ultimately

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[3]This material was provided through the courtesy of Mr. Warren Hintz of the Stephan Company. It consists of the pure grease base without perfume or other additives (Stephan Base No. 3049).

released from the glass after an immersion of approximately 24 hours. Figure 8 shows a typical lagoon wall obtained with clear lacquer (before immersion).

Some success was obtained with the use of epoxy cements on glass surfaces, but lagoon size was very difficult to control. The epoxy plaque, however, was very stable under water and culture media. Most epoxy cements require considerable curing or setting times and are therefore not as convenient to use as greases or quick drying lacquers. With additional experimentation, however, suitable permanently hardening lagoon-field materials should be found, as many compounds of this nature are commercially available.

Stability of Lagoons

As noted previously, the lagoon fields of DCS (and of some of the other grease-like materials) are somewhat unstable on glass when immersed in warm culture medium. This instability appears to be due to the hydrophobic properties of the grease and to a low adhesive affinity for glass, as compared with that of water. The viscosity of the grease also decreases somewhat at the 37° C temperature of incubation and allows rapid release from the surface. The surface tension of the grease at the grease-water-glass intersection apparently acts to peel the grease layer from the surface as illustrated in figure 9(a). The same action tends to break down narrow ridges separating lagoons. (See fig. 9(b).)

Lagoon Depth

The ability of a lagoon to maintain isolation or confinement of cells depends, essentially, upon proper lagoon depth. Unfortunately, lagoon depth is one of the more difficult characteristics to control in lagoon-field formation. It depends in some measure upon the formation material and upon the degree to which the material thickens around the lagoon area during the formation process. In general, both DCS and HCW produce lagoons with depths suitable for most test purposes but, as is discussed subsequently in the section "Monolayering Characteristics and Escape," an occasional cell of certain malignant types does escape after the lagoon becomes filled with cells.

Lagoon depth can be simply determined prior to selection for test purposes by noting the difference in height of the focal planes of two small objects, one on the lagoon bottom surface and the other on the surface of the surrounding grease layer. Cells which have flattened out on these surfaces after vessel inoculation and while some cells are still on the grease are often convenient objects for lagoon depth determinations. Alternately, the objects may be tiny particles (of latex or carbon black) introduced purposely into the culture medium for measuring purposes.

Lagoon depths normally range from 5μ to 20μ for DCS and HCW fields. Rollup of DCS can produce much deeper lagoons, but the lagoon size is also usually large in such cases.

INOCULATION PROCEDURES

General Procedure

The specific technique to be used for inoculation of a micro lagoon field depends upon the nature of the investigation at hand, but for most cell work it consists simply in adding an adequately dilute suspension of cells to the culturing vessel in the usual way and allowing the cells to settle on the field. Some cells will settle directly in the lagoons, usually, but many cells will also settle on the grease surfaces. However, cells settling on DCS or HCW surfaces move about freely on the grease, and most of them eventually enter a lagoon and become entrapped. In a reasonably short time (depending upon the ratio of the total lagoon area to the total field area), practically all cells become confined to lagoons. With sufficiently dilute suspensions, many lagoons can be found which contain only one cell initially; subsequent colonies arising from such cells, of course, constitute true clones. In this way hundreds of isolated clones can be initiated and maintained in a single field.

Specific Culture Vessels and Inoculation Techniques

Petri dishes.- Inoculation of Petri dishes containing micro lagoon fields can be accomplished by addition of the cell suspension and adequate culture medium directly to the dish. Alternately, a more restricted cell field can be obtained by use of the settling cylinder illustrated in figure 10. The cylinder is placed over the preformed lagoon field and securely attached to the vessel surface with a leakproof seal of DCS grease. The cylinder is then filled to the desired depth with the test-cell suspension, and the cells are allowed to settle on the field. After the cells have attached, the cylinder is removed and the dish filled to the proper level with the medium. Settling cylinders of various diameters can be used to obtain the desired degree of surface coverage by cells. Use of a cylinder with a diameter corresponding to that of a given formation signet allows inoculation of only the lagoon field itself.

The settling-cylinder inoculation technique is capable of many useful modifications. For example, the cylinder can be used to exclude cells from a region of the dish by adding the inoculating suspension exterior to the cylinder. Alternately, the regions external and internal to the cylinder can be inoculated simultaneously with different cell lines or with suspensions of different concentrations. In particular, the settling cylinder can be used to inoculate the dish area exterior to a lagoon field with a "feeder-cell" monolayer (refs. 3 and 4) and the lagoons can be used subsequently for clonal growth after inoculation by either dilute suspension or micro manipulator seeding of single cells in the lagoons.[4]

[4]In the present case, however, use of the micro lagoon technique precludes the necessity of using irradiated cells for the feeder-cell layer.

Cooper dishes.- A culturing technique which has been found extremely useful for a variety of purposes consists in the growth of cells within micro lagoons formed on the undersurface of a Cooper dish cover. The field is first formed on the surface and then inoculated by using a settling cylinder, as illustrated in figure 11. After the cells have settled and attached, the suspending medium is emptied and the cylinder removed. The cover is then placed on the dish, which contains a proper level of medium to immerse fully the undersurface of the cover. This arrangement is particularly useful for time-lapse studies where the top mounting of the cells allows direct viewing at a relatively high power with an upright microscope and without the need for special long-working-distance objectives. The technique is also very useful in micromanipulation work where it allows direct and unhindered access to the cells of the lagoon field. This culturing arrangement is much simpler and less time consuming to prepare than conventional perfusion chambers and is directly applicable to most tests where perfusion chambers would normally be used.

By coating the top rim of the Cooper dish with DCS grease to effect a vapor-tight seal upon placement of the cover, these dishes can be maintained for relatively long periods in a dry incubator atmosphere, the grease effectively preventing the evaporation of water from the medium. DCS grease is not impervious to the carbon dioxide used to control the medium pH, however, and this gas must therefore be supplied in an adequate amount. The dish is usually sealed by two small strips of transparent tape at the bottom for ease and safety in handling. The cover may be easily removed for medium changes subsequently by breaking the tape with the thumbnail.

A special mounting plate which has been developed for holding small plastic Cooper dishes securely on the microscope stage for time-lapse and other microscopic observations is illustrated in figure 12. The dish is held securely in a circular recess in the mounting plate by means of two small spring clips, as shown. The dish can be easily snapped into or out of the plate recess by sidewise pressure. With this simple but rigid mounting, plastic Cooper dishes can be used routinely for time-lapse studies of cells in micro lagoons.

It should be noted that by use of two (or more) settling cylinders, all the various inoculation techniques previously described can be advantageously applied with the Cooper dish. (See fig. 13.)

Perfusion chambers.- Use of micro lagoons with perfusion chambers of the type shown in figure 14 is achieved by forming the lagoon field on either of the window cover slips, for upright or inverted microscope viewing. Inoculation is carried out either by use of settling-cylinder techniques prior to assembly or by addition of the cell suspension subsequent to assembly. Care must be exercised in filling such chambers (having grease lagoon fields) to prevent detachment of the lagoon plaque by the medium meniscus in the narrow space between the chamber windows.

Cell Settling Rates and Surface Adhesion

Cell settling rates are important in many aspects of inoculation. The depth of the settling cylinders shown in figures 10, 11, and 13 is 1.5 centimeters. The required time for settling of all cells in a column of suspension of this height is relatively short, being on the order of 50 minutes for most cell types. Free cells in suspension are essentially perfect spheres and hence their settling velocities are accurately estimated by Stokes Law

$$u = \frac{2}{9}\left(\frac{\rho_c}{\rho} - 1\right)\frac{g}{\nu} r^2 \tag{1}$$

provided the cell Reynolds number R lies in the range

$$R = \frac{2}{9}\left(\frac{\rho_c}{\rho} - 1\right)\frac{g}{\nu^2} r^3 \leq 0.1 \tag{2}$$

and provided the cell has no appreciable surface charge. In these relations,

u	terminal settling velocity of cell
ρ_c	cell mass density
ρ	culture medium density
ν	kinematic viscosity of medium
g	gravitational constant
r	cell radius

The ratio ρ_c/ρ is the specific gravity of the cells (relative to the medium) and must, of course, have a value greater than 1.0 for settling to occur. For cells of a given radius and in a given medium, ρ_c/ρ determines whether Stokes Law is valid for the settling process. For values of ρ_c/ρ approaching 1.0, equation (1) is valid for relatively large values of r ($r = 50\mu$). The total settling time T_s for a cell suspension of column height H is

$$T_s = \frac{9}{2} H\left(\frac{\rho_c}{\rho} - 1\right)^{-1}\frac{\nu}{g} r^{-2} \tag{3}$$

An experimentally determined settling-rate curve for mouse fibroblast cells (L-line) is presented in figure 15. A useful formula for determining the resultant

cell-number density σ (cells per unit area of surface after settling) deposited by an inoculation cylinder of height H is given by

$$\sigma = \omega H \tag{4}$$

where ω (cells per unit volume) is the concentration of the inoculation suspension.

Almost as soon as settling cells contact the culturing surface, they attach and begin to flatten out. This flattening process requires approximately 20 minutes for an L-line mouse fibroblast. The cells adhere firmly to either glass or plastic, or even to the grease layer of the lagoon field. The cells move easily over the surface of the grease and, as mentioned previously, ultimately become confined to the lagoons. The surface adhesion of the cells is very strong. Even during mitosis in inverted lagoons (fig. 16) when the cell has rounded up and almost completely released from the surface, the point of contact of the mitotic sphere with the surface is adequate to support the cell against the resultant downward force. Because of this strong adhesion, the Cooper dish cover can be used with inverted lagoons for monolayering and mitotic studies.

Monolayering Characteristics and Escape

In general, cells confined in micro lagoons will undergo continuous growth and mitosis and will ultimately fill the entire lagoon. In the case of normal cells, that is, nonmalignant lines, a full monolayer is formed at which time mitosis ceases or decreases in rate, provided the lagoon walls are of sufficient depth to contain the fully grown monolayer. In the case of malignant cells, containment is more difficult since these cells tend to form multilayers and ultimately overflow the walls of the lagoon. Still, many useful tests can be run with malignant cells in micro lagoons since the cells do not generally tend to escape until the lagoon has become relatively saturated with cells. This is true even in the case of inverted lagoon fields, where it would normally be expected that gravity would favor the escape of the cells down the lagoon wall (fig. 17(a)). However, surface adhesion appears to hold the cells firmly within the walls until they have acquired by growth or multiplication a sufficient depth (fig. 17(b)) to overflow the walls. Occasionally, L-cells have been observed to escape by the attachment of a long pseudopod to some anchoring point on the grease surface and then moving out onto the grease upon subsequent contraction of the pseudopod.

Such escape tendencies, occurring as they do primarily after full monolayering has been achieved, present no difficulties in most test work. However, in cases where long-term studies of full monolayers are desired, care must be taken at the start to select lagoons of adequate depth for the cell type(s) of interest especially when using malignant cells. Lagoon depth may be estimated by use of the procedures previously outlined.

APPLICATIONS AND CONCLUDING REMARKS

The basic usefulness of the lagoon technique lies in applications to experimental investigations in which it is necessary to keep single cells or groups of cells physically confined for observation, without disturbing their normal in vitro metabolic environment. This containment property of micro lagoons has been usefully employed in a number of basic cytological investigations to date.

One of the most important of these uses has been the restriction of test cells to a given microscopic field in time-lapse cinephotographic studies, and it was for this purpose that the technique was originally developed. Here the lagoon walls prevent migration of the cells of interest out of the time-lapse field of view and also prevent intrusion into the field by outside cells. Some of the quantitative time-lapse studies which have been made possible by use of lagoons are determination of cell generation times, durations of mitotic cycle phases, cell growth rates, monolayering dynamics, mitotic synchronization inducements, surface mobilities, and cell contact phenomena. In the case of monolayering studies, the lagoon walls effectively prevent any disturbance of the time-lapse cell field by changes in the number density of cells in distant parts of the culture monolayer due to migrations and cell movements there.

Other applications have involved the use of micro lagoons as isolation chambers for maintaining single cells; thus, response of the cell to various microsurgical and chemical treatments can be followed for relatively long periods without the cell becoming obscured by or mixed with feeder cells of the surrounding culture. Such tests are usually performed directly in the lagoon by a micro manipulator after treatment. By use of the proper settling cylinder arrangement for inoculation, it is possible to grow several cell types in the same culture without direct mixing of the individual populations. On the other hand, the interaction of mixed cell populations can be readily studied by time-lapse methods by using a single lagoon.

Langley Research Center,
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Langley Station, Hampton, Va., August 7, 1968,
129-02-05-05-23.

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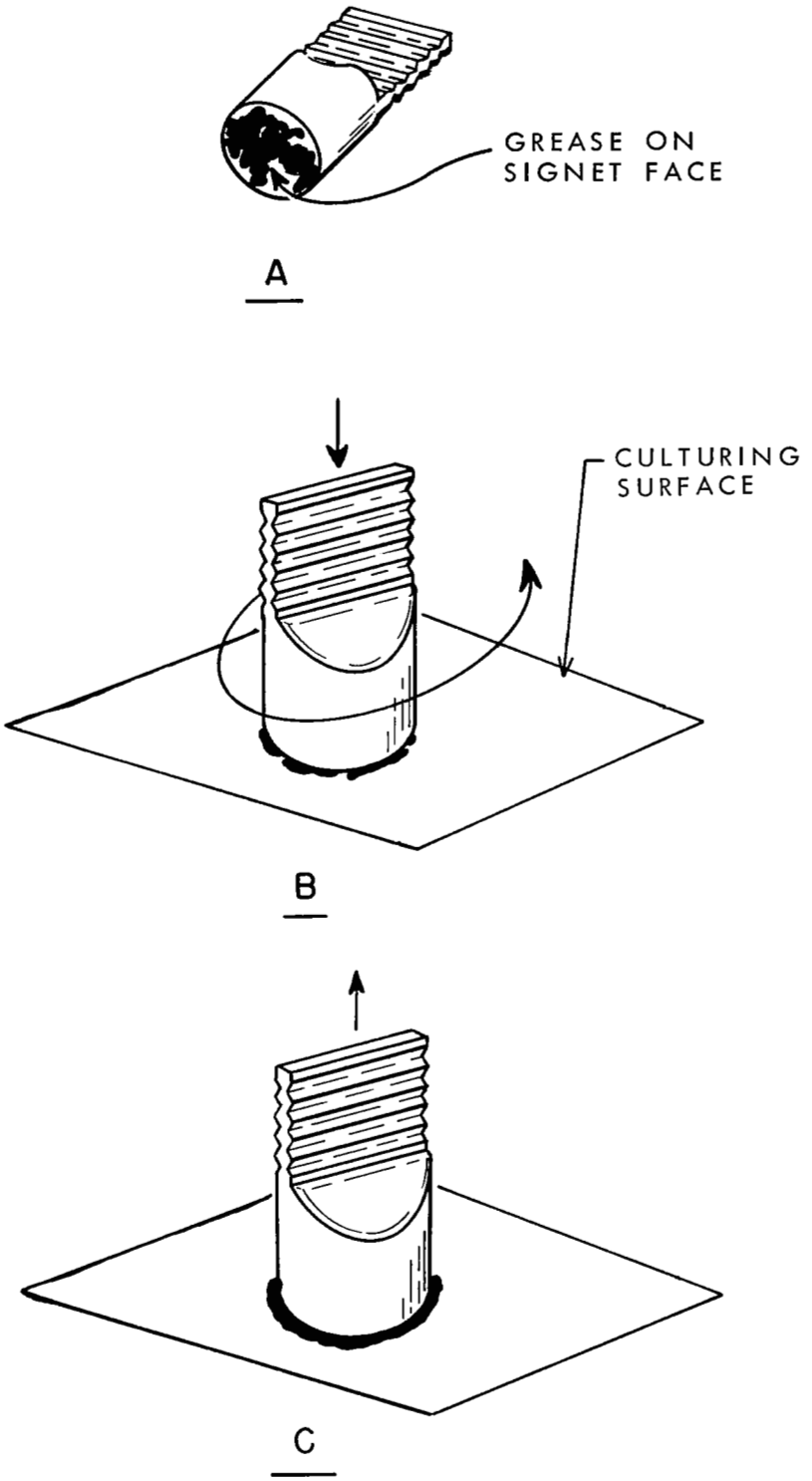


Figure 1.- Procedure for forming a micro lagoon field by using a flat-faced cylinder or "formation signet."

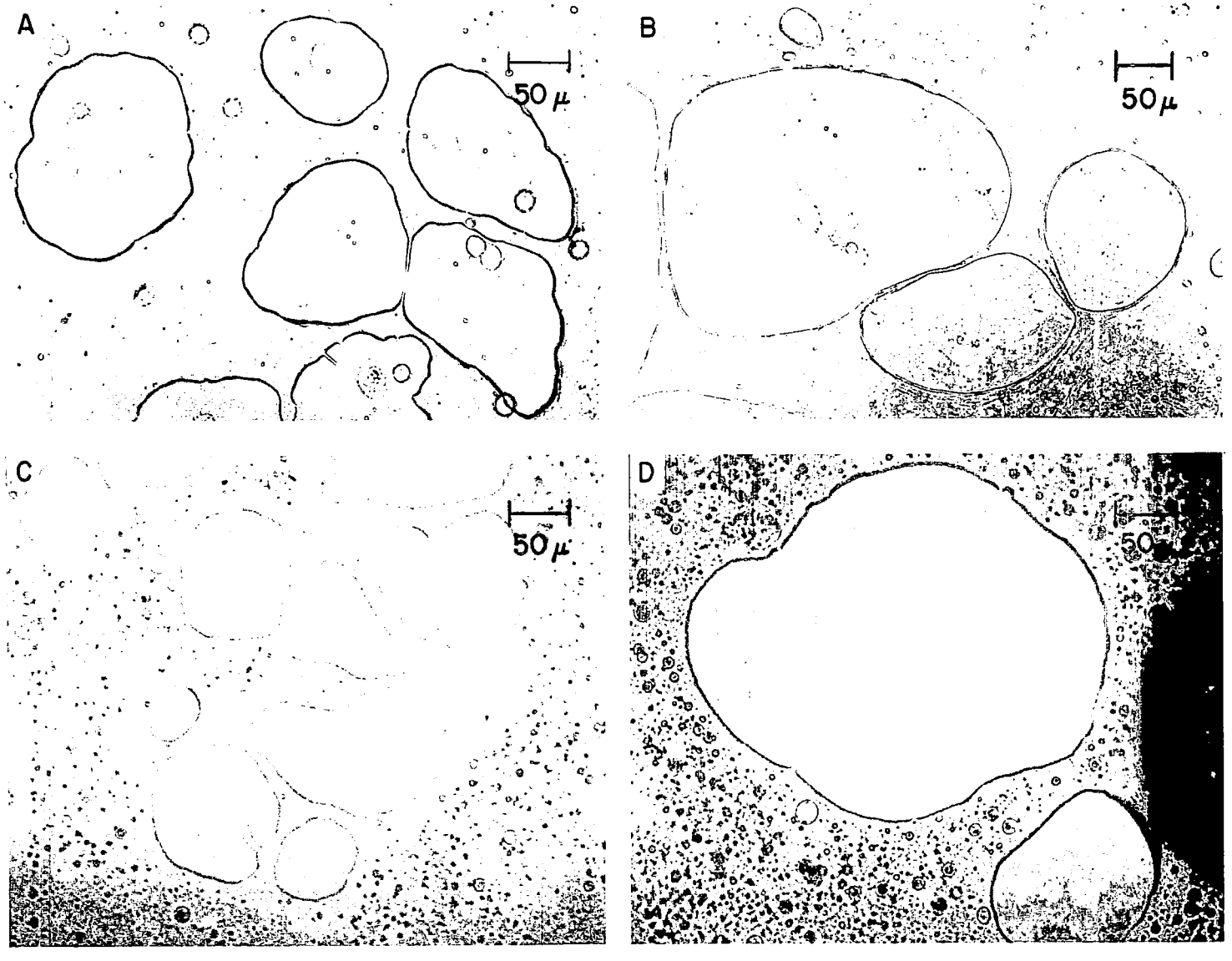
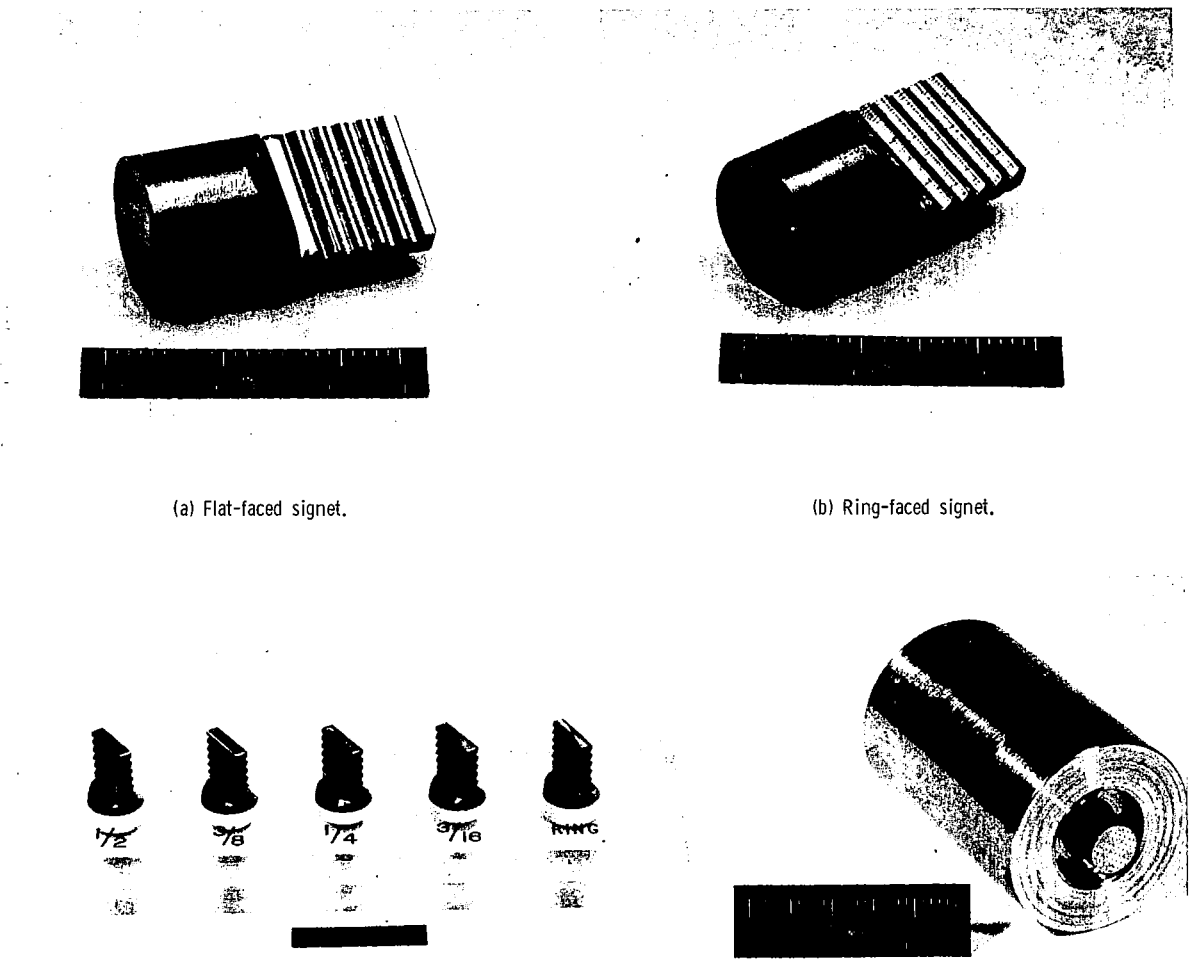


Figure 2.- Typical micro lagoon fields. Micrographs A and B show lagoons containing cells. In micrograph A, the cells are just settling on the surface; in B, the cells have spread in monolayer form.

L-68-5684



(a) Flat-faced signet.

(b) Ring-faced signet.

(c) Family of flat-faced signets of different face diameters.

(d) Grid-faced signet for formation of an ordered lagoon array.

Figure 3.- Lagoon-field formation signets.

L-68-5685

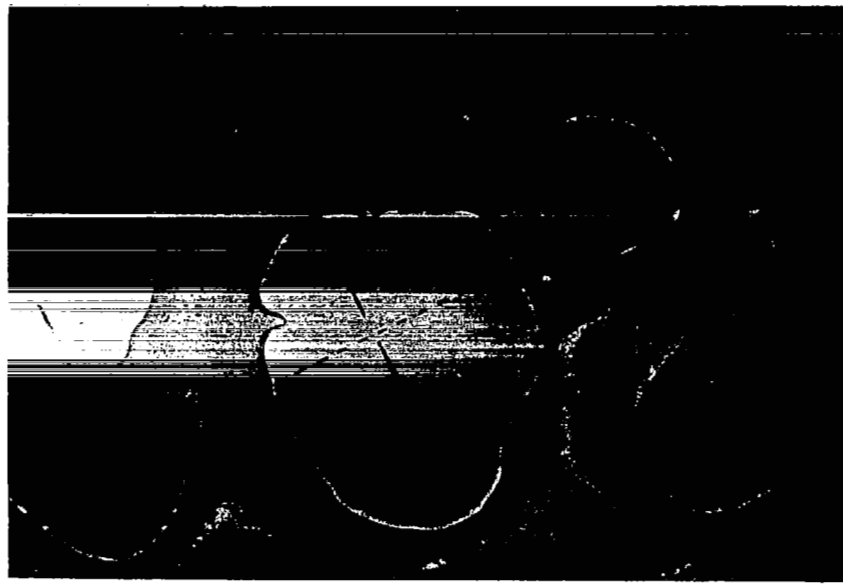


Figure 4.- A typical lagoon field made with grid-faced signet. Calibration, 50μ. L-68-5686

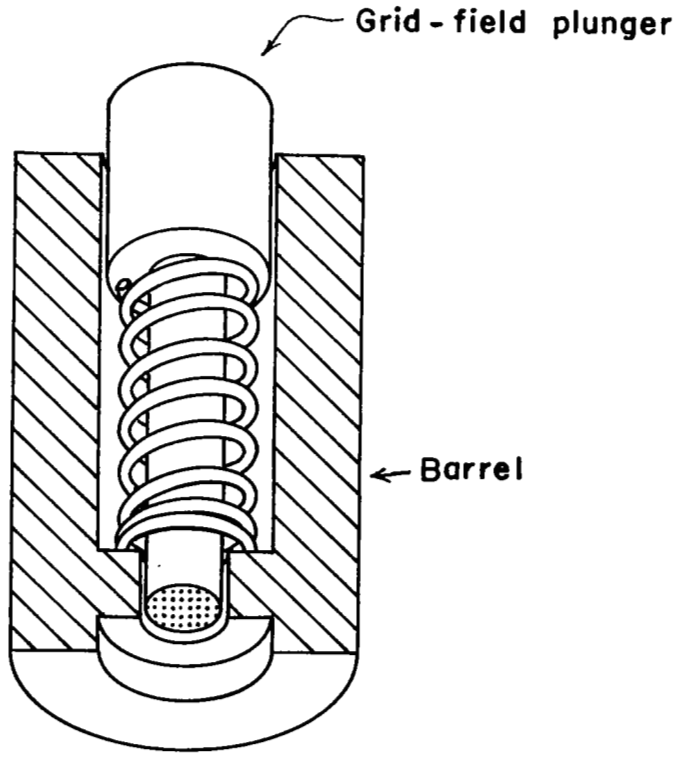


Figure 5.- Grid-faced signet mounted to allow a high degree of reproducibility in lagoon-field formation.

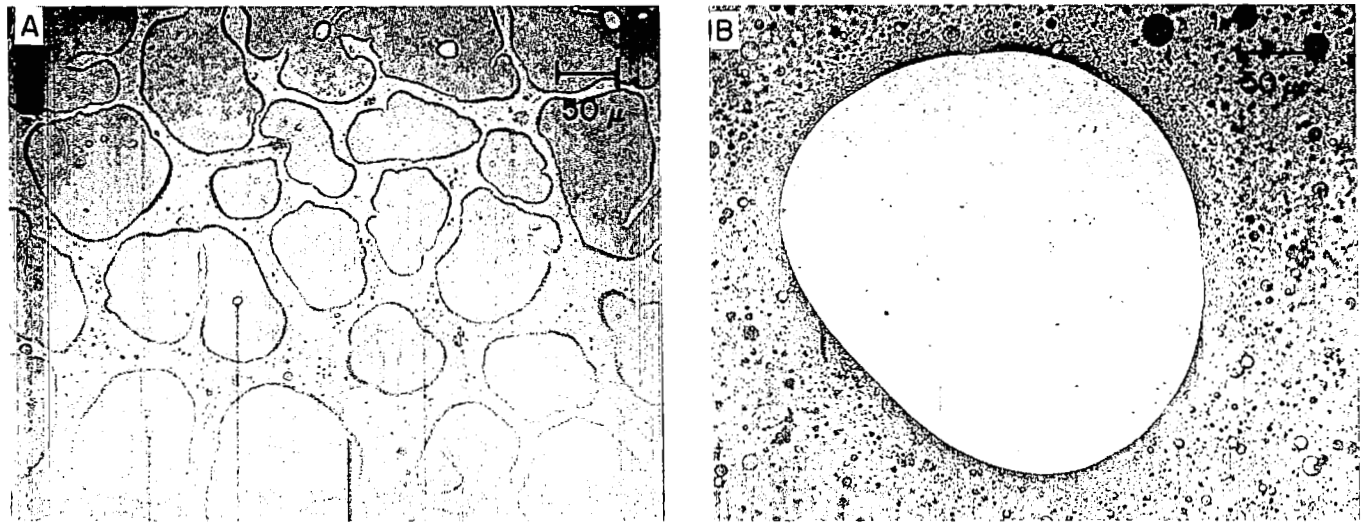


Figure 6.- Typical lagoon formations made of Dow Corning silicone grease (DCS) by using a flat-faced signet. L-68-5687

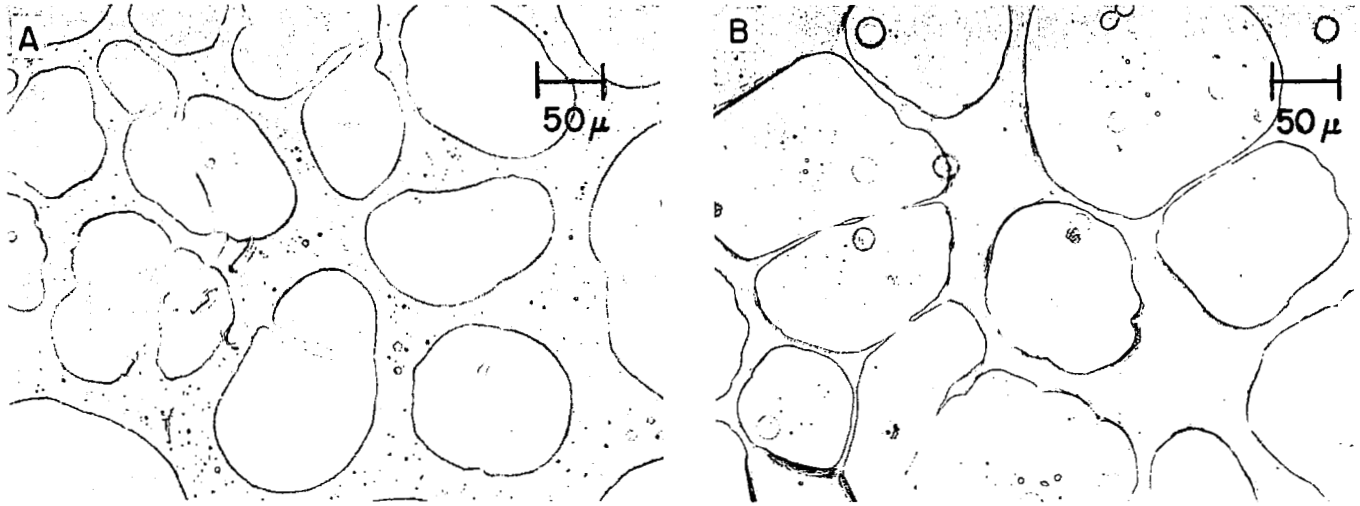


Figure 7.- Typical lagoon formations made of hair control wax (HCW) by using a flat-faced signet. L-68-5688

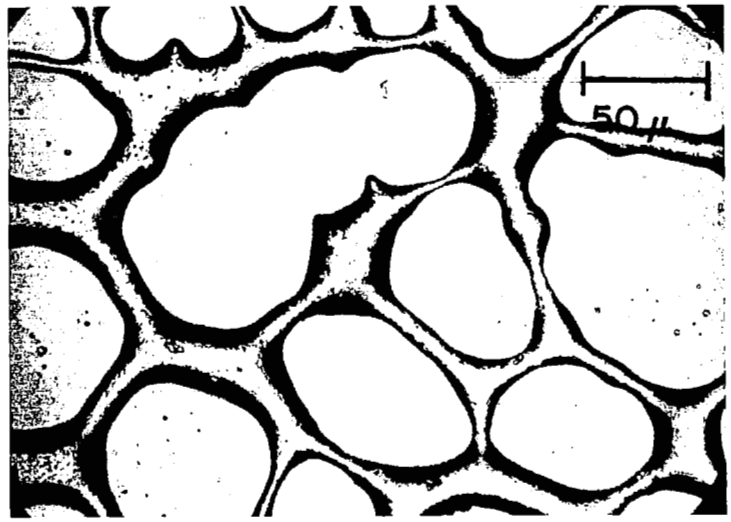
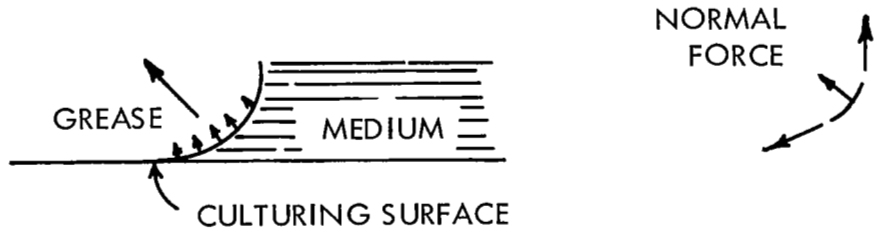
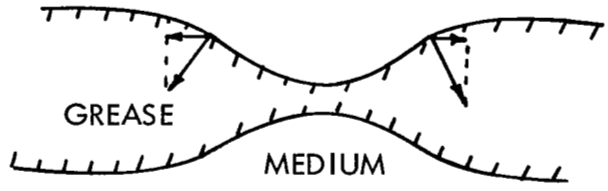


Figure 8.- A typical micro lagoon field formed of lacquer before immersion in culture medium. L-68-5689



SIDE VIEW

(a) Rollback of a lagoon wall.



TOP VIEW

(b) Stretching and breaking of a ridge between two lagoons.

Figure 9.- Illustration of the tendency of grease formation materials to retract under the action of surface tension.

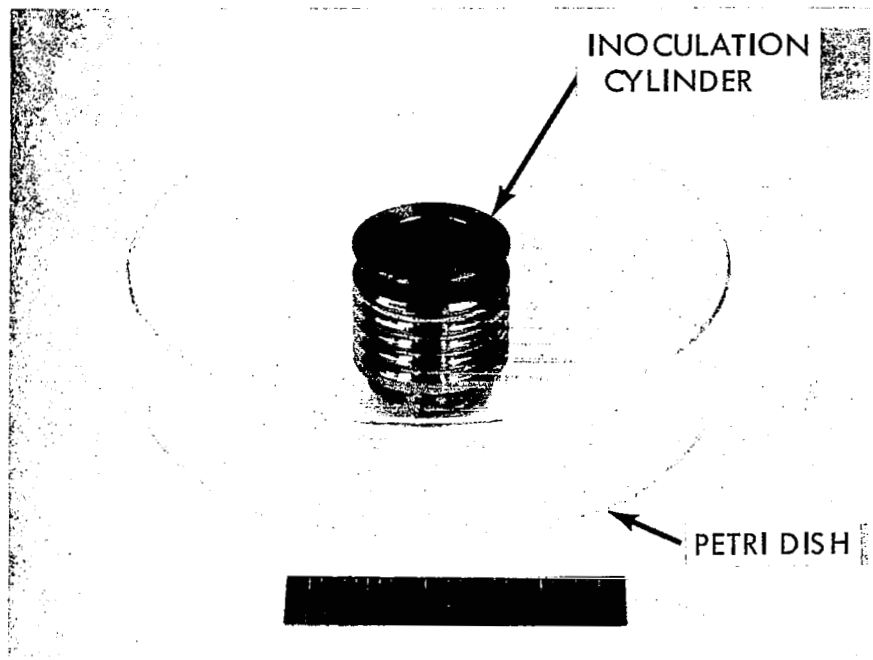


Figure 10.- Inoculation of a micro lagoon field in a plastic Petri dish by using a small settling cylinder. L-68-5690

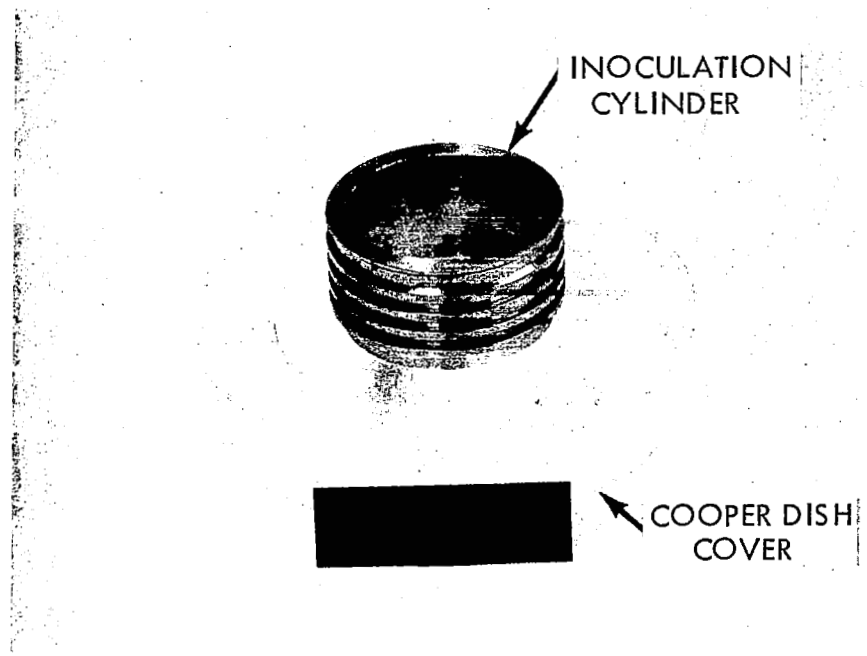


Figure 11.- Inoculation of a Cooper dish cover by using a settling cylinder. L-68-5691

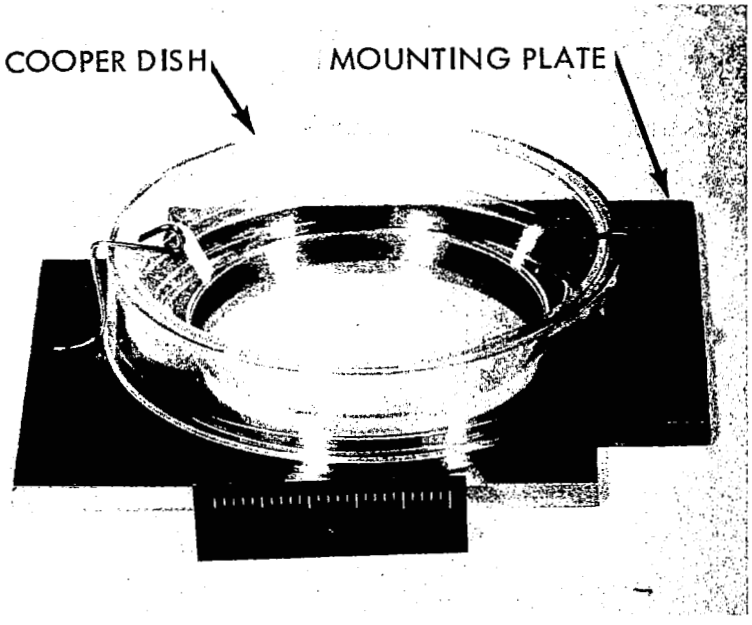


Figure 12.- Special mounting plate for holding plastic Cooper dishes securely for use in time-lapse studies. L-68-5692

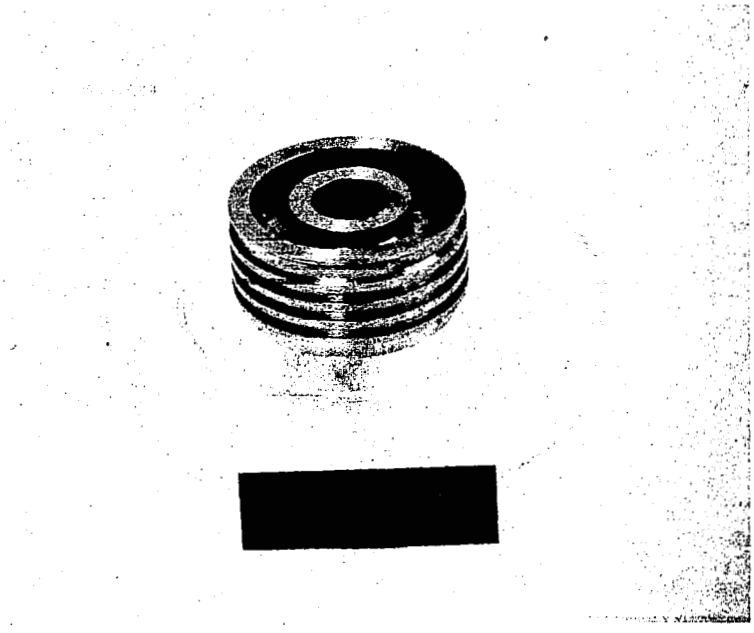


Figure 13.- Inoculation of Cooper dish cover by use of several settling cylinders to obtain separated populations. L-68-5693

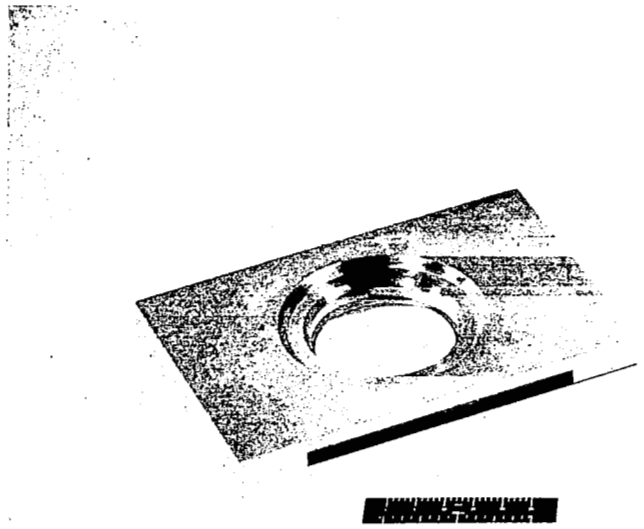


Figure 14.- Stainless-steel perfusion chamber with cover glass windows. L-68-5694

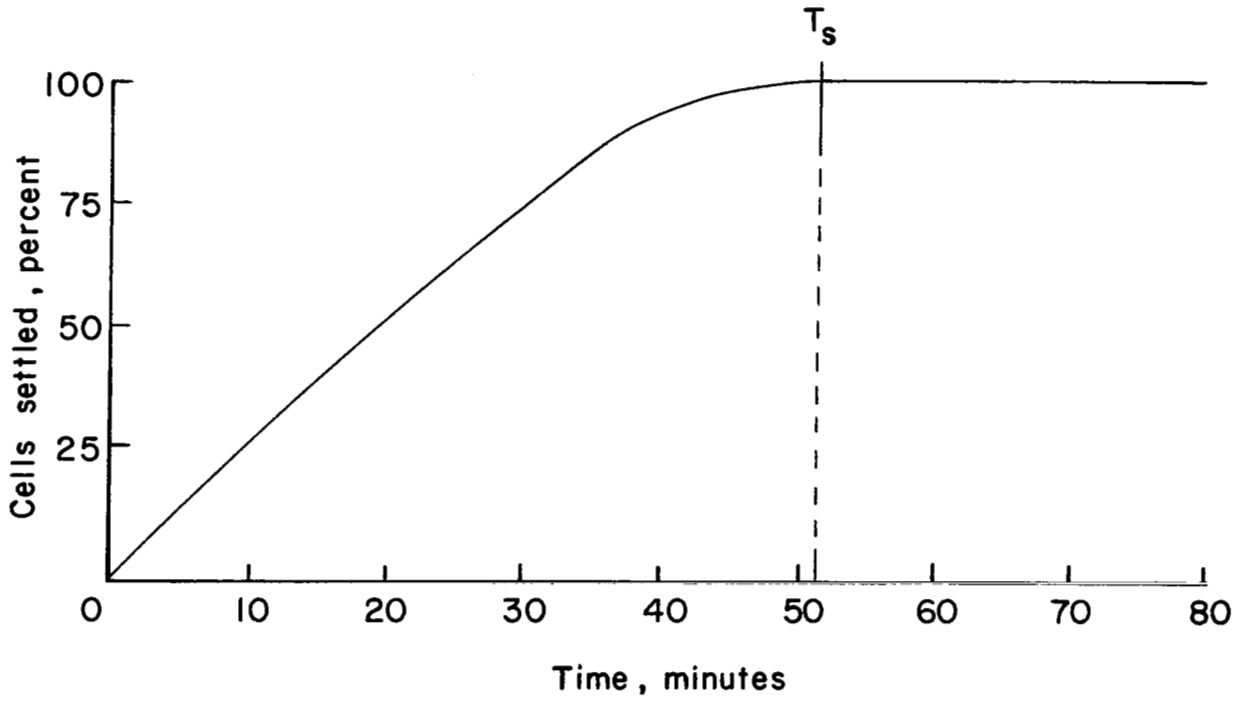


Figure 15.- Plot of settling time T_s for mouse L-strain cells.

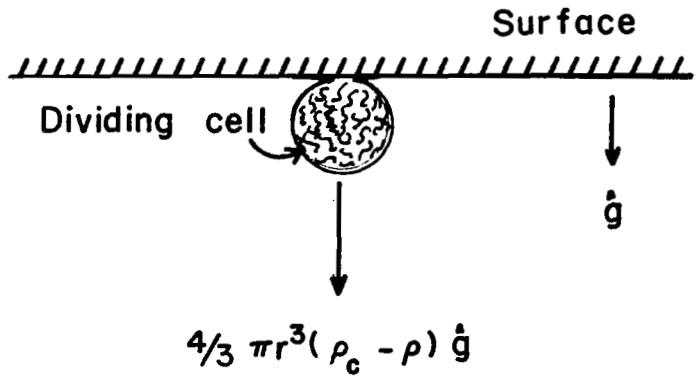
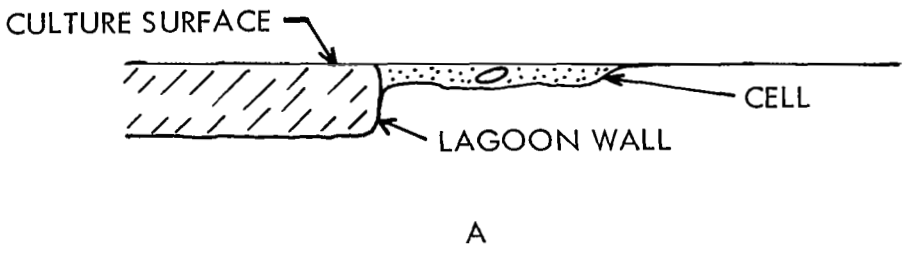
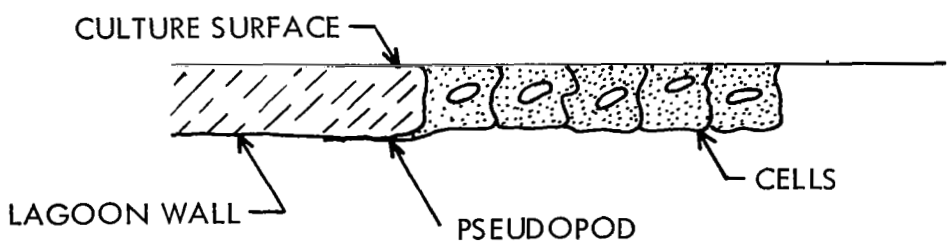


Figure 16.- Rounded-up cell in mitosis adhering to the underside of a Cooper dish cover. The symbol $\vec{g}$ denotes the gravitational acceleration vector.



A



B

Figure 17.- Escape tendency of cells in inverted lagoon fields upon becoming large or tightly packed.